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Molecular Genetic Analysis of Parasite Survival in *P. falciparum* Malaria

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The human malaria parasite *P. falciparum* exhibits extensive strain-dependent chromosomal polymorphisms that have been implicated in the generation of antigenic variability in this organism. These polymorphisms can result in large deletions in chromosomes as determined by pulsed-field gradient gel electrophoresis. We have investigated the molecular basis for extensive deletions in chromosomes 1, 2 and 8 in multiple geographic isolates of this parasite that result in the loss of expression of well-characterized parasite antigens. The structure of these polymorphic chromosomes reveal that a mechanism of chromosome breakage and healing by the addition of telomeric repeats most plausibly accounts for these karyotypes. Furthermore, the orientation of these gene fragments on their truncated chromosomes reveal that the healed chromosome originally associated with centromeric elements is motitically stable and maintained. These studies indicate a specificity to the process by which chromosomal polymorphisms are generated.

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BODY OF REPORT

INTRODUCTION

During the life cycle of the malaria parasite, a series of complex phenotypic switches occur that allow it to alternate between its invertebrate and vertebrate hosts. The majority of these switches occur in a precisely regulated way as morphologic changes during parasite development. Others occur in the form of antigenic variations that contribute to immune evasion by the parasite in the vertebrate host (1). While the molecular basis for most developmental switches is poorly understood, more is known about the antigenic variation observed in this parasite. Extensive chromosomal polymorphisms have been observed in different geographical isolates of the human malaria parasite *Plasmodium falciparum* (2,3). These polymorphisms may reflect a mechanism by which some antigenic diversity arises (4). In addition, the unusual stretches of tandemly repeated protein coding sequences comprising most malarial antigen genes may further facilitate strain variation (5,6,7).

Characterization of polymorphic chromosomes of *P. falciparum* has revealed the presence of large deletions that arise mitotically during the asexual life cycle of the parasite, both *in vivo* and *in vitro* (3,4,8). In addition, nonparental karyotypes have been identified after a genetic cross of two cloned isolates indicating that chromosomal polymorphisms also arise meiotically (9,10). It has been observed that large chromosomal deletions are associated with the loss of specific antigens (11,12). This observation has facilitated the characterizaiton of these chromosomes and the mechanism by which these deletions arise. The first such rearrangement to be described was in the gene for the knob-associated histidine-rich protein (KAHRP) (4), which maps to chromosome 2. This protein is required for the formation of knobs (13), structures that mediate the binding of the infected erythrocyte to capillary endothelial cell surfaces (14,15,16). As a consequence of knob formation and endothelial cell binding, high parasitemias and obstruction of the micro-circulation are observed that contribute to the virulence associated with falciparum malaria (17). Spontaneous mutants have been isolated that have lost the knob and are thus avirulent (18). We have previously shown that loss of the knob in these mutants resulted from a rearrangement in the gene for the KAHRP with deletion of 3' sequences and association of the truncated gene with a telomere (4).

We have used the KAHRP rearrangement, which generates the knobless phenotype as a model for chromosomal polymorphisms, and have characterized the structure of the polymorphic chromosome 2 in four independent K^- isolates. An extensive deletion was found on those chromosomes distal to the KAHRP gene, and an abrupt transition occurred at the breakpoint from coding sequence to telomeric repeats. No evidence of a target for homologous recombination was found, suggesting that chromosome breakage and healing by the addition of telomeric repeats has occurred. Further support for this mechanism in generating deletions was found in the analysis of a chromosome 8 polymorphism that was associated with the loss of HRP II gene expression. In contrast to the KAHRP, the functions

of the other histidine-rich proteins of *P. falciparum*, HRP II and III are unknown. HRP III is nonessential to survival of the parasite in its vertebrate and insect hosts (9); HRP II is dispensible in culture and may be nonessential *in vivo* as well. Characterization of chromosome 8 from an HRP II$^-$ isolate revealed a structure similar to what was found for the chromosome 2 polymorphisms in the KAHRP gene. These results suggest that the mechanism of DNA breakage and telomere healing in generating these polymorphisms may be general. This process may also suggest a mechanism by which changes in Plasmodial gene expression may be occurring, such that the widely observed chromosome polymorphisms may indicate other changes in specific gene expression.

RESULTS

Rearrangements on chromosome 1 in the RESA gene indicate a specificity for chromosome breakage and healing in *P. falciparum* (Pologe, de Bruin and Ravetch, submitted 12/89)

Ring-infected erythrocyte surface antigen-negative isolates of *Plasmodium faiciparum* demonstrate a complex DNA rearrangement with inversion of 5' coding sequences, deletion of upstream and flanking sequences, and healing of the truncated chromosome by telomere addition. An inversion intermediate that results in the telomeric gene structure for RESA has been identified in the pathway. This inversion created a mitotically stable substrate for the sequence-specific addition of telomere repeats at the deletion breakpoint.

The genome of *Plasmodium falciparum*, the protozoan parasite responsible for the most severe form of human malaria, is extremely flexible. It displays polymorphisms on the order of fifty to hundreds of kilobase pairs between genetically equivalent chromosomes from different parasite isolates(12,4,3). These chromosome length polymorphisms can involve deletions in which structural genes are lost (19,4,20). One well-characterized mechanism by which this class of polymorphisms is generated is the introduction of double-stranded breaks in the DNA, followed by the enzymatic addition of telomere repeats to the free 3' ends, thereby stabilizing the foreshortened chromosome fragment to mitosis (20). A conserved sequence element has been identified at the breakpoint of multiple, independent events, suggesting a specificity to either the breakage or the healing reaction (20). This specificity has been further characterized by the detailed analysis of a subtelomeric rearrangement for chromosome 1 in the ring-infected erythrocyte surface antigen (RESA) gene.

RESA is a 155,000-M, peptide of unknown function which is deposited onto the erythrocyte surface by the invading merozoite during asexual development (21) and which accumulates in the erythrocyte cytoplasm surrounding the gametocyte at the onset of sexual development (22). The structure of the RESA gene in a RESA$^-$ isolate has been shown to involve both an inversion of the signal-encoding exon and a large deletion. This inversion is mediated by homologous recombination between homopolymeric A and T sequences in the 5' untranslated and intron sequences, respectively. The deletion includes DNA sequences from

the middle of the signal exon 5' to the end of the chromosome. The resulting truncated gene is followed by an abrupt transition to the *P. falciparum* telomere repeat sequence (23).

CONCLUSIONS

These studies indicate that an inversion intermediate can be detected and is likely to precede the deleted, healed form. A previous model (23) for generating the RESA$^-$ gene structure which proposed an unresolved recombination event with nuclease digestion and healing instead of an intermediate is therefore incorrect. The inversion appears to be a required first step in this DNA rearrangement. If specificity resided in the cleavage reaction, then healing could generate a RESA$^-$ telomeric gene in the absence of inversion. We have never detected a RESA$^-$ telomeric gene with the signal exon in an uninverted oritentation. The inversion rearranges the conserved CA dinucleotide to a centromere-proximal position. Breakage and healing of the inverted sequence then result in a mitotically stable, truncated chromosome. These results indicate a sequence specificity for the healing reaction, which always includes a CA dinucleotide.

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Large Deletions Result from Breakage and Healing of P. falciparum Chromosomes

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Summary

The human malaria parasite P. falciparum exhibits extensive strain-dependent chromosomal polymorphisms that have been implicated in the generation of antigenic variability in this organism. These polymorphisms can result in large deletions in chromosomes as determined by pulsed-field gradient gel electrophoresis. We have investigated the molecular basis for extensive deletions in chromosomes 2 and 8 in multiple geographic isolates of this parasite that result in the loss of expression of well-characterized parasite antigens. The structure of these polymorphic chromosomes reveal that a mechanism of chromosome breakage and healing by the addition of telomeric repeats most plausibly accounts for these karyotypes. Furthermore, the orientation of these gene fragments on their truncated chromosomes reveal that the healed chromosome originally associated with centromeric elements is mitotically stable and maintained. A model for the possible role of this mechanism in the complex parasite life-cycle is discussed.

Introduction

During the life cycle of the malaria parasite, a series of complex phenotypic switches occur that allow it to alternate between its invertebrate and vertebrate hosts. The majority of these switches occur in a precisely regulated way as morphologic changes during parasite development. Others occur in the form of antigenic variations that contribute to immune evasion by the parasite in the vertebrate host (Howard and Barnwell, 1984). While the molecular basis for most developmental switches is poorly understood, more is known about the antigenic variation observed in this parasite. Extensive chromosomal polymorphisms have been observed in different geographical isolates of the human malaria parasite Plasmodium falciparum (Kemp et al., 1985; Van der Ploeg et al., 1985). These polymorphisms may reflect a mechanism by which some antigenic diversity arises (Pologe and Ravetch, 1986). In addition, the unusual stretches of tandemly repeated protein coding sequences comprising most malarial antigen genes may further facilitate strain variation (Cowman et al., 1985; Sharma et al., 1985; Galinski et al., 1987).

Characterization of polymorphic chromosomes of P. falciparum has revealed the presence of large deletions that arise mitotically during the asexual life cycle of the parasite, both in vivo and in vitro (Van der Ploeg et al., 1985; Pologe and Ravetch, 1986; W. Trager, personal communication). In addition, nonparental karyotypes have been identified after a genetic cross of two cloned isolates indicating that chromosomal polymorphisms also arise meiotically (Walliker et al., 1987; Sinnis and Wellems, 1988). It has been observed that large chromosomal deletions are associated with the loss of specific antigens (Wellems et al., 1987; Kemp et al., 1987). This observation has facilitated the characterization of these chromosomes and the mechanism by which these deletions arise. The first such rearrangement to be described was in the gene for the knob-associated histidine-rich protein (KAHRP; Pologe and Ravetch, 1986), which maps to chromosome 2. This protein is required for the formation of knobs (Kilejian, 1979), structures that mediate the binding of the infected erythrocyte to capillary endothelial cell surfaces (Trager et al., 1966; Luse and Miller, 1971; Udeinya et al., 1983). As a consequence of knob formation and endothelial cell binding, high parasitemias and obstruction of the microcirculation are observed that contribute to the virulence associated with falciparum malaria (Miller, 1969). Spontaneous mutants have been isolated that have lost the knob and are thus avirulent (Langreth and Peterson, 1985). We have previously shown that loss of the knob in these mutants resulted from a rearrangement in the gene for the KAHRP with deletion of 3' sequences and association of the truncated gene with a telomere (Pologe and Ravetch, 1986).

We have used the KAHRP rearrangement, which generates the knobless phenotype as a model for chromosomal polymorphisms, and have characterized the structure of the polymorphic chromosome 2 in four independent K^- isolates. An extensive deletion was found on those chromosomes distal to the KAHRP gene, and an abrupt transition occurred at the breakpoint from coding sequence to telomeric repeats. No evidence of a target for homologous recombination was found, suggesting that chromosome breakage and healing by the addition of telomeric repeats had occurred. Further support for this mechanism in generating deletions was found in the analysis of a chromosome 8 polymorphism that was associated with the loss of HRP II gene expression. In contrast to the KAHRP, the functions of the other histidine-rich proteins of P. falciparum, HRP II and III are unknown. HRP III is nonessential to survival of the parasite in its vertebrate and insect hosts (Walliker et al., 1987); HRP II is dispensible in culture and may be nonessential in vivo as well. Characterization of chromosome 8 from an HRP II^- isolate revealed a structure similar to what was found for the chromosome 2 polymorphisms in the KAHRP gene. These results suggest that the mechanism of DNA breakage and telomere healing in generating these polymorphisms may be general. This process may also suggest a mechanism by which changes in Plasmodial gene expression may be occurring, such that the widely observed chromosome polymorphisms may indicate other changes in specific gene expression.

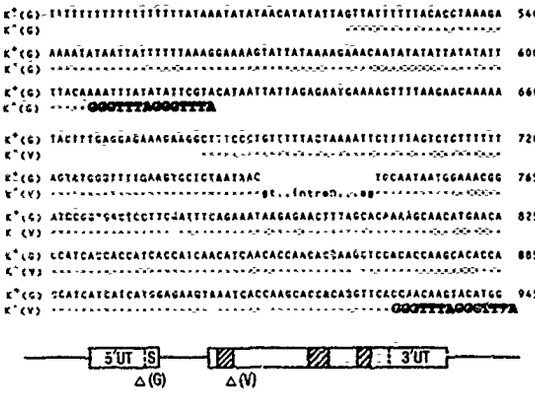

Figure 1. Comparison of the Wild-Type KAHRP Gene and the FVO⁻, D3, D4, and E96 Mutant Gene Sequences

(+) indicates knobby. (−) indicates knobless. K⁺(G) indicates the pertinent region of the FCR3 cDNA sequence (Pologe et al , 1987) K⁻(G) indicates sequences obtained from clones D3, D4, and isolate E96 (see text). K⁻(V) corresponds to the FVO⁻ sequence, which was obtained from a genomic clone and contains an intron after position 748. The conserved splice donor and acceptor sequences are indicated in lower case. The 435 bp intron has been omitted for the sake of clarity. Dashed lines in the K⁻ sequences indicate identity to the wild-type KAHRP cDNA sequence. Telomere sequences are accentuated by a boldface font. A map of the wild-type KAHRP gene is presented below the sequences. Open rectangles indicate transcribed sequences 5′ and 3′ untranslated (UT) sequences, the signal (S) sequence, and repeat sequences (hatching) are shown The positions of the breakpoints in the Gambian (G) and Vietnam (V) K⁻ genes are indicated

Results

A Complex Rearrangement Is Responsible for the Loss of KAHRP Gene Expression in K⁻ Mutants

We had previously shown that the breakpoint of the deletion in the KAHRP gene of chromosome 2 differed in two geographic isolates. The breakpoint in the Vietnam isolate FVO⁻ occurred in the polyhistidine-encoding sequences, while the breakpoint in the Gambian isolates D3 and D4 occurred in the 5′ untranslated sequence (Pologe and Ravetch, 1986). Characterization of the sequences at the breakpoint was pursued to elucidate the molecular basis for this deletion. A genomic clone was obtained from the FVO⁻ isolate after brief Bal31 digestion of total genomic DNA and was subjected to DNA sequence analysis. The sequence obtained is shown in Figure 1 and is compared with the previously published cDNA sequence for the KAHRP gene (Pologe et al., 1987). The FVO⁻ sequence is identical to the FCR3 cDNA sequence (K⁺) from the XmnI cloning site at nucleotide 681 up to 748, where an intron of 435 nucleotides interrupts the genomic sequence. Identity resumes at nucleotide 749 and continues to the deletion breakpoint at nucleotide 930 at which point an abrupt transition is seen to the telomeric repeat GGGTTTA. Variation in this telomeric repeat was observed after five copies of this sequence, demonstrating the variant sequence GGGTTCA or the less frequent sequences GGTTTA and GGGTTCCA. Approximately 1 kb of telomeric sequences were determined (data not shown) from this genomic FVO⁻ clone with no evidence of either unique sequence or subtelomeric repeats.

To determine the breakpoint sequences from the Gambian K⁻ clones D3, D4, and E96, an alternate approach was employed. Amplification of the sequences surrounding the breakpoints was accomplished in a polymerase chain reaction (PCR) using the thermostable Taq DNA polymerase (Saiki et al., 1988). The derived sequences demonstrated a single breakpoint for all three Gambian K⁻ clones (Figure 1). The common breakpoint for D3 and D4, which are independently cloned K⁻ isolates from a mixed FCR3 population, suggest that the mutational event preceeded the cloning of these isolates. However, E96, a spontaneous K⁻ mutant that arose in the clonal K⁺ population A2 (derived from FCR3), is the result of an independent mutational event. Sequence alignment of this breakpoint with the wild-type sequence (Figure 1) demonstrates a similar abrupt transition at nucleotide 665 from KAHRP-encoding sequences to the telomeric repeat sequence. The K⁻ mutation arises spontaneously in culture in mitotically growing parasites and can be isolated from in vivo infections (Trager, personal communication; Corcoran et al., 1986). This phenotype is nonviable in vivo, resulting in the death of the parasite in the spleen of its host (Langreth and Peterson, 1985). Persistance of a mechanism that results in a nonviable phenotype suggested that it may serve an alternative function in the parasite. We therefore sought to determine if this mechanism is of more general significance in generating chromosomal polymorphisms.

A Structurally Similar Deletion of Chromosome 8 Occurs in the HRP II Gene in an HRP II⁻ Strain

The HRP II gene maps to chromosome 8, which demonstrates strain-dependent polymorphism, as seen in Figure 2A. HRP II mRNA expression is undetectable in strain D10 (data not shown). This strain also demonstrates a more rapidly migrating chromosome than the HRP II⁺ strains A2, FCR3, D3, and HB3. The association of a null phenotype with a chromosomal polymorphism suggested that it might demonstrate a deletion similar to the KAHRP on chromosome 2. To determine if this in fact is the case, DNA isolated from D10 and an HRP II⁺ isolate were digested with the restriction endonuclease BglII and probed with the HRP II cDNA probe. As seen in Figure 2B, two DNA fragments are detected with this probe: the HRP II fragment migrating at 7.2 kb and the cross-hybridizing HRP III fragment of 23 kb. In the HRP II⁻ mutant D10, the HRP II fragment appears smaller and diffuse, diagnostic of the heterogeneity of telomere-containing fragments. The relationship of the HRP II gene to the telomere of chromosome 8 was evaluated in the mutant and wild-type strains by Bal31 sensitivity. As seen in Figure 2C, the HRP II gene in the D10 isolate is sensitive to the processive exonuclease activity of this enzyme, while the wild-type strain is resistant to Bal31 digestion under identical conditions (data not shown). Thus, the DNA rearrangement in strain D10 places the gene closer to a chromosome end. Detailed restriction mapping of the HRP II gene in the HRP

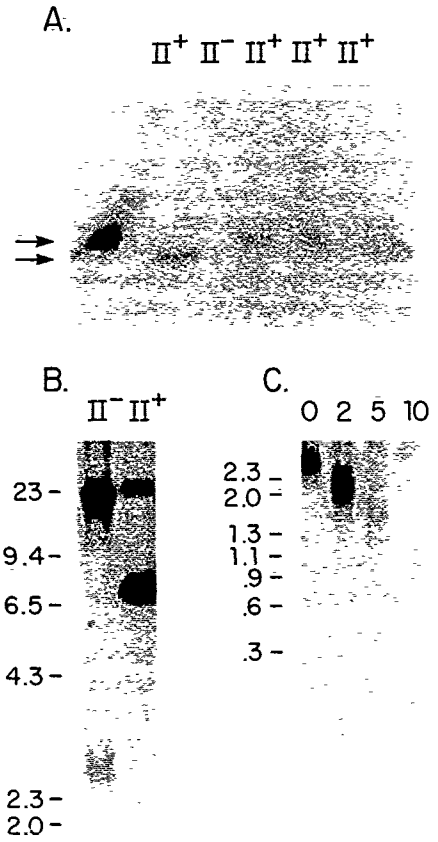

Figure 2. Genomic Organization of the HRP II Gene in II+ and II− Isolates

(A) Chromosome mapping of the HRP II gene. P. falciparum strains are A2, D10, FCR3, D3, and HB3 (left to right). II+ and II− indicate wild-type or mutant for HRP II gene expression. Little cross-hybridization is detected to the HRP III gene on chromosome 13 (Kemp et al., 1987) under the conditions of DNA transfer and hybridization used here.

(B) Southern blot analysis of the HRP II gene. BglII digest of DNA isolated from D10 (II−) and FCR3 (II+) probed with the HRP II cDNA clone 6412 under moderate stringency conditions (final wash 55°C). Molecular weight markers (kb) are shown on the left.

(C) Bal31 susceptibility of the HRP II gene in HRP II− isolate D10. Numbers at the top of each lane indicate digestion time in minutes. After Bal31 treatment, DNA aliquots were digested with BglII, fractionated onto 1% agarose Tris-acetate EDTA gels, and probed with the HRP II cDNA probe.

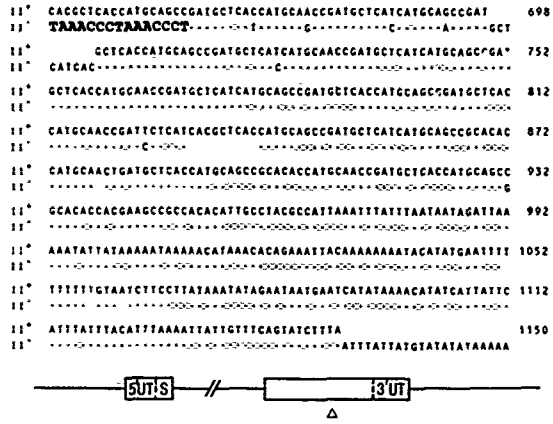

Figure 3. Comparison of the Wild-Type and Mutant HRP II Gene Sequences

The HRP II+ sequence was derived from the 7G8 isolate and the HRP II− sequence from strain D10. Dashed lines indicate sequence identity. The telomere repeat sequence is indicated in boldface. Nucleotide numbers correspond to those of the published 7G8 sequence (Wellems and Howard, 1986). Differences between the two sequences all occur within the coding sequence of the gene. They include several silent third base substitutions, three substitutions that result in amino acid changes, an insertion, and a deletion, all of which presumably reflect strain-dependent sequence variation (Ravetch et al., 1985). A map of the HRP II+ gene is drawn below the sequence comparison. The position of the HRP II− breakpoint is indicated by the arrowhead.

II+ isolates and the D10 strain demonstrates that a deletion has occurred in the 5′ coding sequence of the rearranged gene with the remaining 3′ sequences associated with a telomere.

The precise structure of this rearranged chromosome was determined by molecularly cloning the HRP II− gene from the D10 isolate. The sequence of that clone was determined and compared with wild-type HRP II sequence from the Brazilian isolate 7G8 (Wellems and Howard, 1986), as seen in Figure 3. The sequences are nearly identical from nucleotide 659 to the 3′ end of the published sequence (position 1150). The C-rich strand of the canonical telomere repeat is immediately adjacent to the 5′ end of the HRP II sequence, establishing the polarity of this gene on chromosome 8 relative to its centromere. The orientation of the KAHRP and HRP II genes on their respective chromosomes relative to the centromere is illustrated in Figure 4, where it can be seen that the two genes are in opposite polarity relative to their respective centromeres.

Comparison of the sequences occurring at the breakpoint of chromosomes 2 and 8 involved in these polymorphisms is shown in Figure 4. A six-out-of-eight nucleotide homology was found flanking the breakpoint in two of the three sequences determined. This sequence was absent in the D3, D4, and E96 breakpoints in the KAHRP gene apart from a CA dinucleotide immediately preceeding the breakpoint. The significance of these sequences in mediating breakage or healing will require additional examples to be adequately assessed. No evidence was found for an extensive target that might mediate homologous recombination between telomeric sequences and internal chromosomal sequences.

Discussion

We have defined the structure of two polymorphic chromosomes that contain large deletions of DNA and result in altered gene expression. The simplest mechanism that accounts for the data presented here is one in which DNA breakage occurs, followed by healing of the broken ends by the addition of telomeric repeat sequences. While healing by recombination has been extensively characterized in yeast (Dunn et al., 1984; Haber and Thorburn, 1984),

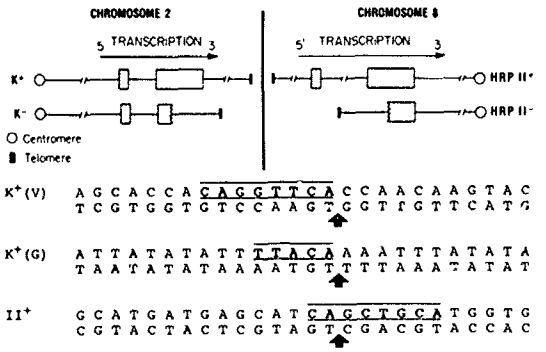

Figure 4 Summary of the KAHRP and HRP II Gene Structures

(Top) Summary maps of the chromosome 2 and 8 deletions The transcriptional orientations of the KAHRP and the HRP II genes on chromosomes 2 and 8, respectively, are indicated with arrows above each map. Open rectangles represent transcribed sequences in the unrearranged genes Single lines indicate nontranscribed sequences Broken lines indicate undetermined distances

(Bottom) Summary of wild-type sequences flanking the breakpoints in the KAHRP and the HRP II genes All sequences are presented from centromere to telomere (left to right) across the breakpoint The arrows indicate the positions of the breakpoint Limited homologies are overlined The HRP II sequence is compiled from the D10 isolate 5' of the breakpoint and from the 7G8 sequence following the breakpoint (Wellems and Howard, 1986) The conserved CA sequence immediately preceding the breakpoint comes from the rearranged D10 gene

it is less likely that interchromosomal homologous recombination between sequences that are internal on one chromosome and telomeric on another could generate the structures we have characterized. The reciprocal product predicted in this type of mechanism has not been detected nor is a substantial homology to telomeric sequences present near the breakpoints of these deletions. Sequences flanking the breakpoints have not been detected elsewhere in the genome (L Pologe and J V Ravetch, unpublished data). This lack of a target for homologous recombination similarly argues against a mechanism of intrachromosomal recombination. The recent suggestion that recombination mediated by a 21 base repeat (rep 20) located in a subtelomeric position can account for these polymorphisms (Corcoran et al., 1988) is unlikely since no evidence of a rep 20 sequence is found at or near the breakpoints of these chromosomes.

Chromosome breakage and healing by the addition of telomeric repeat sequences has been described in other lower eukaryotes (Greider and Blackburn, 1985; Blackburn, 1986) and can result in distal deletions (Forney and Blackburn, 1988). Chromosome healing can be envisioned to occur on both broken fragments, with loss of the fragment not originally associated with a centromeric element upon subsequent rounds of mitotic growth. This model can account for the large distal deletions observed in the polymorphic chromosomes described here and for the observed polarity of the gene fragments relative to their respective centromeres.

It has been observed that the K^+ phenotype is stable in some P. falciparum clonal isolates while other isolates give rise to K^- mutants spontaneously upon propagation in vitro, which accumulate as a result of their growth advantage (W. Trager, personal communication). This indicates that the mechanism of chromosome breakage and healing occurs during mitotic growth. The frequency of K^- appearance varies in different strains, suggesting that the mechanism that generates these phenotypes may be a mutation of a wild-type mechanism and not a consequence of a normal pathway. This would further suggest that other genetic loci may be contributing to the chromosomal rearrangements that result in the K^- phenotype. It is intriguing to note that the chromosome structures described here that result in the K^- and HRP II^- phenotypes are indistinguishable from the chromosome structure of a mutant in P. tetraurelia, which results in the loss of the A surface antigen gene (Forney and Blackburn, 1988). In that ciliated protozoan, DNA breakage and healing occur in a developmentally regulated fashion during the generation of the somatic macronucleus from the germ-line micronucleus This results in the active transcription of macronuclear genes. In the d48 mutation, an aberrant breakage event occurs within the transcriptional unit for the A gene that results in its partial deletion. The resulting truncated gene is found immediately adjacent to telomeric repeats. We suggest that like the d48 mutation, the K^- and HRP II^- gene structures may be the result of an aberrant mechanism. A normal counterpart for this mechanism may exist in Plasmodia which could result in the regulation of specific gene classes by changing their chromosomal positions relative to the telomere (Borst and Greaves, 1987). Characterization of other chromosomal polymorphisms will determine if gene activation can occur as a consequence of breakage and healing.

Experimental Procedures

Parasites

P. falciparum isolates D3 and D4 were cloned by micromanipulation of the Gambian isolate FCR3 (Trager et al , 1981) Isolate E96 is a K^- mutant that arose spontaneously in the clonal K^+ isolate A2 (W Trager, personal communication) All FCR3-derived isolates were kindly supplied by Dr William Trager Isolate FVO^- was cloned by limiting dilution from the Vietnam clone FVO^+ (Gritzmacher and Reese, 1984) and was provided by Dr Robert Reese Isolate D10 was cloned by limiting dilution from strain FC27, which was originally isolated from a patient in Papua New Guinea (Anders et al , 1983, Corcoran et al , 1986) and was generously provided by Dr Thomas E. Wellems Parasites were grown in synchronous culture as described (Trager and Jensen, 1976, Pasvol et al , 1978, Lambros and Vanderberg, 1979)

DNA Isolation, Bal31 and Restriction Enzyme Digestions, Gel Electrophoresis, and DNA Hybridization

DNA was obtained from P. falciparum isolates as described by Goman et al (1982) Restriction enzymes and Bal31 were used according to the manufacturer's specifications (NEB) Radiolabeled cDNA probes were prepared by nick-translation with [α-^{32}P]dATP (Maniatas et al , 1982) Hybridization of cDNA probes was under stringent conditions (50% formamide, 10% dextran sulfate, 5x SSC, 7 mM Tris [pH 7 6], 1x Denhardt's solution, 25 μg/ml of salmon sperm DNA, at 42°C) Southern blots were washed at 55°C or 65°C in 0 1x SSC and 0 1% SDS Hybridization with 5' end-labeled oligonucleotides were performed as described by Zeff et al (1986) Pulsed field gradient gel electrophoretic separation of P falciparum chromosomes was performed using the method of Schwartz and Cantor (1984) Agarose blocks containing P falciparum-infected erythrocytes at 10%–20% parasitemias were pre-

pared as previously described (Van der Ploeg et al , 1985) Chromosomes were separated in 1% agarose, 0 5× TBE gels electrophoresed at 150 V at 10°C for 36 hr at a pulse frequency of 300 sec in both the north-south and east-west directions Prior to transfer to nitrocellulose (Southern, 1975), gels were soaked in 0 25 M HCl for 1 hr.

Taq Polymerase Amplification

Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer They were deacetylated by NH_4OH treatment at 55°C, lyophilized, resuspended in distilled water, and either used directly or purified by reverse phase HPLC on a C18 column as recommended by Applied Biosystems The primers for the Taq polymerase reaction were oligonucleotide 215, which corresponds to nucleotides 236–255 in the KAHRP gene sequence, oligonucleotide 328, which is complementary to nucleotides 520–539 in the wild-type gene and contained a BamHI recognition sequence on the 5′ end, and the telomere oligonucleotide 130 $(CCCTAAA)_3$ Thirty cycles of DNA amplification were carried out in reaction mixtures containing 1 μg of total genomic DNA as the starting template and 1 μg each of the oligonucleotide primers 215 and 130 in 100 μl reaction volumes containing 2.5 U of Taq polymerase (NEB), 16 6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8 8), 6 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0 2 mM each of dATP, dCTP, dGTP, dTTP, and 170 μg/ml of BSA The reaction cycle was 1 5 min annealing at 45°C, 3 min of extension at 72°C, and 1 5 min of denaturation at 94°C The last cycle consisted of a 7 min extension reaction. To prepare labeled DNA fragments for Maxam-Gilbert sequencing, the amplified products were extracted extensively with phenol followed by chloroform and then ethanol-precipitated Oligonucleotide 328 was 5′ end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (NEB) and used as a primer with 10% of the amplified target DNA in a single extension reaction (1 5 min denaturation, 94°C, 2 min annealing, 44°C, 7 min extension, 72°C)

DNA Sequencing

DNA sequencing was carried out by the dideoxy chain-terminating method using total plasmid as the starting template and synthetic oligonucleotide primers (Sanger et al , 1977) and by the chemical degredation method (Maxam and Gilbert, 1980)

HRP II cDNA Cloning

cDNA clones for the HRP II gene of P falciparum strain FCR3 were isolated by oligonucleotide hybridization A 48 base degenerate oligonucleotide was constructed to the repeat sequence of the published HRP II gene from the Brazilian clone 7G8 (Wellems and Howard, 1986) The oligonucleotide was end labeled with [γ-^{32}P]ATP and used to screen a cDNA library constructed to trophozoite-enriched RNA from the Gambian isolate FCR3 (Kochan et al , 1986) Three clones were isolated Clone 6412 is approximately 800 bp Partial sequencing demonstrated 223 bp of 5 untranslated sequence and approximately 580 bp of coding sequence

Telomere Cloning

The KAHRP$^-$ gene was molecularly cloned from P falciparum K$^-$ isolate FVO$^-$ and the HRP II$^-$ gene was isolated from P falciparum clone D10 Since the mutant KAHRP and HRP II genes were located near chromosome ends, telomere containing libraries were prepared by the method of Van der Ploeg et al (1984) Total genomic DNA was briefly pretreated with the processive exonuclease Bal31 (NEB) to unblock chromosome ends rendering them accessible to cloning enzymes Any recessed 3′ ends were repaired with DNA polymerase, Klenow fragment (NEB) For KAHRP$^-$ cloning, the Bal31-treated FVO$^-$ DNA was digested with Xmn-1 The DNA was ligated into SmaI digested pUC19 that had been dephosphorylated with bacterial alkaline phosphatase The ligation mixture was transformed into E coli MC1061 and screened with the nick-translated KAHRP cDNA clone LP20 (Pologe et al , 1987) Plasmids were prepared from 13 positive clones and used to transform the recombination deficient E coli strain DH 5 1 Three stable clones were isolated that differed in the number of telomere repeats, ranging from 1 25 kb to 1 75 kb of telomere DNA Similarly, for HRP II$^-$ gene isolation, blunt-ended Bal31 treated D10 DNA was restricted with BglII and ligated asymetrically into BamHI–SmaI cut pUC19 A library was generated upon transformation into E coli MC1061, and screened with the nick-translated HRP II cDNA insert from clone 6412 One stable positive clone was isolated containing a 1 8 kb insert and analyzed in detail

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Note Added in Proof

A chromosome 1 polymorphism has been characterized in strains A2, D3, D4, and E96 and found to be the result of breakage within the signal sequence of the RESA gene, with deletion of all 5′ sequences and healing of the broken end The sequences at the break point resemble those found for chromosome 2K$^+$(G) isolates D3, D4, and E96 (Figure 4), further confirming the generality of this mechanism in P falciparum

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A and T Homopolymeric Stretches Mediate a DNA Inversion in *Plasmodium falciparum* Which Results in Loss of Gene Expression

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Ring-infected erythrocyte surface antigen-negative isolates of *Plasmodium falciparum* demonstrate a complex DNA rearrangement with inversion of 5′ coding sequences, deletion of upstream and flanking sequences, and healing of the truncated chromosome by telomere addition. An inversion intermediate that results in the telomeric gene structure for RESA has been identified in the pathway. This inversion creates a mitotically stable substrate for the sequence-specific addition of telomere repeats at the deletion breakpoint.

The genome of *Plasmodium falciparum*, the protozoan parasite responsible for the most severe form of human malaria, is extremely flexible. It displays polymorphisms on the order of fifty to hundreds of kilobase pairs between genetically equivalent chromosomes from different parasite isolates (8, 10, 14). These chromosome length polymorphisms can involve deletions in which structural genes are lost (4, 10, 11). One well-characterized mechanism by which this class of polymorphisms is generated is the introduction of double-stranded breaks in the DNA, followed by the enzymatic addition of telomere repeats to the free 3′ ends, thereby stabilizing the foreshortened chromosome fragment to mitosis (11). A conserved sequence element has been identified at the breakpoint of multiple, independent events, suggesting a specificity to either the breakage or the healing reaction (11). This specificity has been further characterized by the detailed analysis of a subtelomeric rearrangement for chromosome 1 in the ring-infected erythrocyte surface antigen (RESA) gene.

RESA is a 155,000-M_r peptide of unknown function which is deposited onto the erythrocyte surface by the invading merozoite during asexual development (2) and which accumulates in the erythrocyte cytoplasm surrounding the gametocyte at the onset of sexual development (13). The structure of the RESA gene in a RESA$^-$ isolate has been shown to involve both an inversion of the signal-encoding exon and a large deletion. This inversion is mediated by homologous recombination between homopolymeric A and T sequences in the 5′ untranslated and intron sequences, respectively. The deletion includes DNA sequences from the middle of the signal exon 5′ to the end of the chromosome (see Fig. 2). The resulting truncated gene is followed by an abrupt transition to the *P. falciparum* telomere repeat sequence (3).

In order to determine the mechanism and generality of the RESA gene deletion and chromosome 1 rearrangement, restriction mapping was performed on DNAs from a number of *P. falciparum* isolates. Figure 1 shows *Hin*cII-digested genomic DNA probed with a RESA sequence probe which recognizes the 5′-most *Hin*cII RESA gene fragment. A 7.8-kilobase fragment was detected in the DNA from RESA$^+$ isolate FC27, as well as in DNAs from clone D10 and the nonclonal isolate FCR3. However, five FCR3-derived clones displayed a smaller *Hin*cII band and migrated as heterogeneous-sized DNA fragments. BAL 31 susceptibility of the RESA gene in total genomic DNA from one of these clones demonstrated that the gene had assumed a telomeric location in these parasites (E96, data not shown). Cloning and sequencing of the D3 mutant confirmed the previously reported RESA$^-$ gene structure and demonstrated the identical breakpoint (3, data not shown). It is

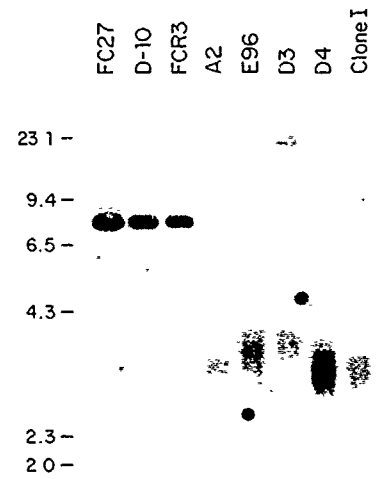

FIG. 1. Southern blot analysis of the RESA gene in DNA from multiple isolates. FC27 (RESA$^+$) is a mixed isolate derived from a patient in Papua New Guinea (9). Isolate D10 (RESA$^+$) was cloned by limiting dilution from strain FC27 (1, 8). FCR3 (RESA$^+$) was isolated from the blood of a patient from Gambia (7). Clones A2, D3, D4, and clone I (RESA$^-$) were cloned by micromanipulation from the Gambian isolate FCR3 (12). E96 (RESA$^-$) is a K$^-$ isolate that arose spontaneously from the clonal K$^+$ isolate. A2 (W. Trager, personal communication). *Hin*cII-digested DNA was probed under stringent hybridization conditions with a DNA fragment that spanned nucleotides 1919 to 1984 in the published RESA sequence (5). Sizes are shown on the left, in kilobases.

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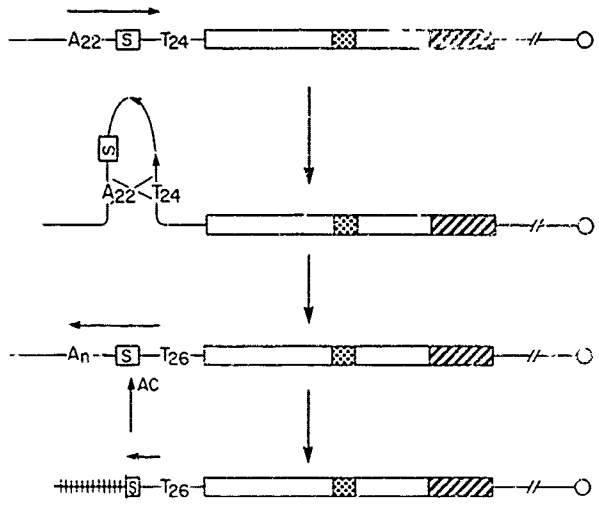

FIG. 2. Proposed mechanism for generating the RESA⁻ structure. The RESA gene is located on chromosome 1 and is oriented 5′ to 3′, telomere to centromere. Symbols: ○, centromere; //, indeterminant number of kilobase pairs; ▭, translated sequences of the RESA gene with the nontranscribed poly(dA) and poly (dT) sequences 5′ of the signal encoding sequences (S) and second exon, respectively; ▭ and ▨, sequences encoding repeated amino acids (the direction of transcription is 5′ to 3′ from left to right); → (above the signal exon), sequence that is inverted in the pathway; +++++, telomere repeats. The conserved CA at the breakpoint is shown in line 3 oriented 5′ to 3′, centromere to telomere.

likely, therefore, that the differences in the mobilities of the RESA⁻ *Hin*cII fragments in Fig. 1 are due to differences in the average number of telomere repeats in the various parasite populations and not to differences in the deletion breakpoints. The breakpoint occurs following a CA dinucleotide sequence, as has been found immediately preceding the transition to telomere repeats in every gene rearrangement to a telomere in *P. falciparum* described to date (11). This suggests that a common sequence element is necessary for some step in the pathway to a viable rearrangement (Fig. 2). This observation leads us to propose a model in which an inversion, which is mediated by base pairing and a crossover event between the homopolymeric A and T sequences that flank the inverted sequences, precedes the deletion event. Subsequent DNA breakage (Fig. 2, ↑ arrow) and telomere addition generated the observed chromosome 1 structure.

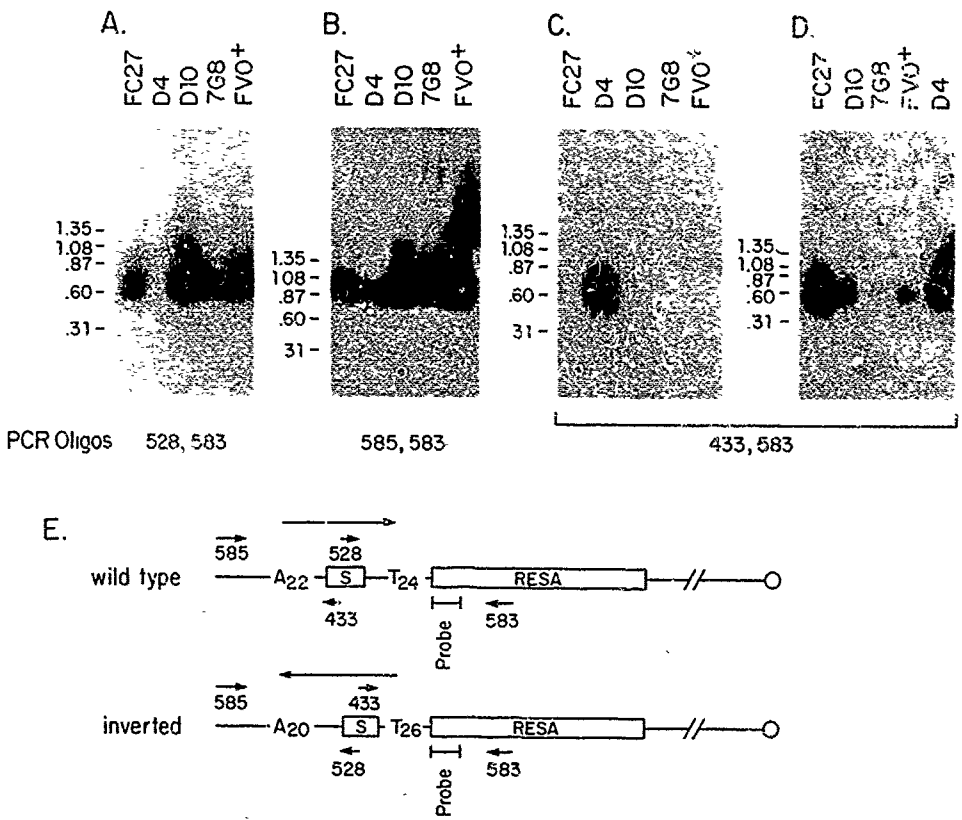

FIG. 3. PCR analysis of wild-type and rearranged RESA sequences from total genomic DNA purified from RESA⁺ isolates FC27, D10, 7G8, and FVO⁺ and RESA⁻ isolate D4. (A) DNA amplification of wild-type RESA sequences. (B) DNA amplification of wild-type and inverted intermediate sequences. (C) DNA amplification of inverted sequences, including sequences organized to a telomere (exposure, 1 h, 70°C with an intensifying screen). (D) overexposure (2 days, −70°C with an intensifying screen) of panel C with D4 amplified DNA loaded onto the gel at a 1:100 dilution compared with other samples. (E) map of RESA gene (top) and inversion intermediate (bottom) on chromosome 1 with PCR oligonucleotides indicated in their 5′-to-3′ orientation. Blots were probed as described elsewhere (15). Oligonucleotide sequences are 585 (TTAAAAGCTTCCTTATTCTTG), 528 (CTCAACAATATATGGGTAC), 433 (CTATATGCATGAAAAGGTCTC), 583 (AAGTTATCAACCTTTTCG), and probe (CCATTATATCCAAGATTACCC). Other details of the map are described in the legend to Fig. 2.

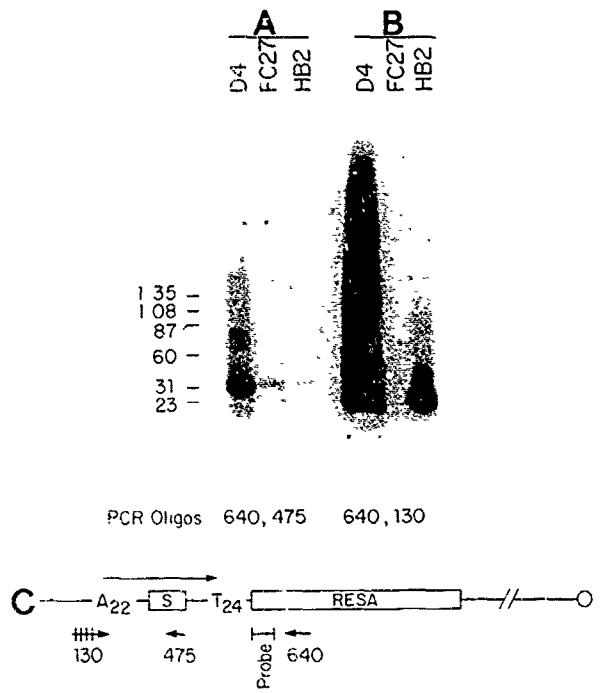

FIG. 4. (A and B) PCR analysis of rearranged RESA sequences from RESA⁻ isolate D4 and RESA⁺ isolates FC27 and HB2. Oligonucleotides (Oligos) used to target sequences for amplification are indicated under the autoradiogram. (A) Inverted sequences; (B) sequences rearranged to a telomere. (C) Map of the RESA gene on chromosome 1 with PCR oligonucleotides and other details as described in the legend to Fig. 3. Oligonucleotide 475 (TATAGTAT TCCAATAATTGG) was used with oligonucleotide 640 (CTGCA GAAGCTTGAACATCTATCAGTAAA) to specifically amplify the inversion. Oligonucleotide 130, containing three copies of the C-rich telomere repeat sequence CCCTAAA, and oligonucleotide 640 were used to specifically amplify RESA sequences that had been rearranged to a telomere.

We have described similar rearrangements involving chromosome breakage and healing by telomere addition for the knob-associated histidine-rich protein gene on chromosome 2 and the histidine-rich protein II gene on chromosome 8 (11).

This model predicts the existence of an intermediate in which the signal exon and the intron sequences are inverted. To identify this intermediate, the polymerase chain reaction (PCR) was used with oligonucleotides which would specifically target the inverted structure. Total genomic DNAs from a RESA⁻ mutant and from a number of parasite isolates in which the RESA gene appeared unrearranged were characterized.

Oligonucleotides which would amplify only the wild-type sequence (528,583), the inverted sequence (433,583), or both (585,583) were synthesized (Fig. 3E). The PCR-amplified products were detected by hybridization with an internal sequence (probe). Figure 3A shows the amplification of wild-type RESA sequences in five different geographical isolates. A 580-base-pair fragment was detected in all RESA⁺ isolates but was absent in the RESA⁻ isolate D4. Even upon extreme overexposure of the autoradiograms, there is no evidence of wild-type RESA sequences in this isolate. However, using oligonucleotides which flank the presumptive inverted sequences and which would therefore target both the wild-type and the presumptive inversion intermediate, we amplified a 950-base-pair DNA fragment from all of the parasite isolates tested including the D4 DNA (Fig. 3B). This observation has been verified by using a more internal 5′-flanking oligonucleotide, which generates a correspondingly smaller inversion fragment in RESA⁻ strain D4 of the same intensity, as seen here (data not shown). To confirm the presence of an inversion in strain D4, oligonucleotides that amplify only the inversion were used (433,583; Fig. 3C). Oligonucleotide 433 corresponds to a DNA segment that is not deleted in the telomeric gene. Hybridization with an internal ^{32}P-labeled oligonucleotide detects an abundant product from the RESA⁻ D4 DNA that corresponds to the gene in the telomeric configuration as well as to a putative inversion intermediate. Significantly, the presence of this inversion is also detected with very long exposure times in DNA from wild-type parasite populations (Fig. 3D). We chose to investigate a number of geographically distinct isolates (FC27 and D10, Papua New Guinea, 7G8, Brazil, D4, Gambia; FVO⁺, Vietnam) to verify the generality of the RESA inversion.

In order to discriminate between the putative inversion intermediate and RESA sequences that were inverted and healed by telomere addition, oligonucleotide 475 was employed. This oligonucleotide corresponds to sequences that are deleted in the RESA⁻ telomeric gene and can amplify sequences of only an inverted, undeleted intermediate. Specific amplification of the inversion intermediate, which resulted in a 290-base-pair fragment in RESA⁻ isolate D4 and at a diminished level in the RESA⁺ isolates FC27 and HB2, was observed (Fig. 4A). Amplification of the inverted, telomere-associated DNA fragment was accomplished through the use of an oligonucleotide primer which corresponds to the telomere sequence and an internal RESA sequence (130,640). Amplification of the telomere-associated sequences in these three isolates resulted in an abundant species that was derived from the major RESA species in the D4 DNA, which migrates as a smear because of the amplification of many hundreds of telomeres. Significantly, the HB2 isolate displayed a minor population of sequences in which the RESA gene is associated with a telomere; no telomerically located RESA gene was found in DNA from the FC27 isolate.

These studies indicate that an inversion intermediate can be detected and is likely to precede the deleted, healed form, as predicted in the model proposed above (Fig. 2). A previous model (3) for generating the RESA⁻ gene structure which proposed an unresolved recombination event with nuclease digestion and healing instead of an intermediate is therefore incorrect. The inversion appears to be a required first step in this DNA rearrangement. If specificity resided in the cleavage reaction, then healing could generate a RESA⁻ telomeric gene in the absence of inversion. We have never detected a RESA⁻ telomeric gene with the signal exon in an uninverted orientation. The inversion rearranges the conserved CA dinucleotide to a centromere-proximal position. Breakage and healing of the inverted sequence then result in a mitotically stable, truncated chromosome. These results indicate a sequence specificity for the healing reaction, which always includes a CA dinucleotide. Whether similar sequence specificity is required for the breakage event is still undetermined.

These results demonstrate a novel pathway by which a gene can be inactivated in *P. falciparum*. The generality of this mechanism is currently under investigation. DNA inversion has been shown to regulate gene expression in *Salmonella typhimurium* and bacteriophage Mu. Inversion of pro-

moter-containing segments in *S. typhimurium* switches between the production of two types of flagellar antigens (16), whereas inversion of 3′ coding sequences outside the promoter in bacteriophage Mu modulates the expression of tail fiber protein genes and thereby broadens its host range (6). It is intriguing to speculate that signal exon inversions mediated by flanking homopolymers of A and T, perhaps including transcriptional control signals, may occur as a biological switch to modify gene expression in *P. falciparum*.

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MINIREVIEW

Chromosomal Polymorphisms and Gene Expression in *Plasmodium falciparum*

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RAVETCH, J. V., 1989. Chromosomal polymorphisms and gene expression in *Plasmodium falciparum*. *Experimental Parasitology* 68, 121–125.

It has long been appreciated that the human malaria parasite, *Plasmodium falciparum*, demonstrates extensive strain-dependent polymorphisms with regard to antigenic determinants and protein isoforms. This extensive strain variation is both a theoretical and a practical barrier to the development of strategies to intervene in the life cycle of this parasite. The genetic basis for some of this polymorphism has been elucidated over the past several years, with the application of molecular biological approaches to the characterization of the parasite. As a consequence of these studies, the extent of genetic polymorphisms demonstrated by this parasite has become increasingly apparent, indicative of a genomic plasticity not previously appreciated. The structural basis for this genetic variability and its functional consequences are briefly reviewed here.

The parasite alternates between two hosts during its complex life cycle. During its asexual stage the parasite is a haploid organism, propagating first mitotically within the liver and then within the erythrocyte of its vertebrate host. Differentiation into male and female gametocytes occurs during the erythrocytic stage of growth. Ingestion of these organisms by the feeding mosquito initiates the sexual cycle of development, with fertilization between gametes occurring within the insect vector, followed by meiosis during early division of the zygote. It is during the sexual cycle that independent assortment of chromosomes occurs to generate novel karyotypes. Recombination between genes has been observed and can occur by either homologous or unequal crossing-over, generating novel chromosomes.

Chromosome Structure and the Evidence for Polymorphism

Analysis of the chromosomes of *P. falciparum* has been hampered until recently by the failure of these structures to condense and be easily visualized. Separation of chromosome-sized DNA molecules by pulsed-field gradient electrophoresis (PFG) for *P. falciparum* blood stage parasites revealed discrete molecules, ranging in size from 800 to 3500 kb (Van der Ploeg *et al.* 1985; Kemp *et al.* 1985). Accurate sizing of the chromosomes was established by restriction enzyme analysis of the isolated molecules with comparison of the resultant fragments to bacteriophage λ concatamers (Wellems *et al.* 1987). It is now reasonably well established that 14 discrete chromosomes can be reproducibly identified (Wellems *et al.* 1987; Kemp *et al.* 1987), accounting for the genome size of 2.5×10^7 bp. This value is in good agreement with the number of kinetochores visualized by electron microscopy (Prensier and Slomianny 1986). The asexual erythrocytic stage of the parasite is haploid (Walliker *et al.* 1987),

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with no gross chromosomal changes apparent during the stages of erythrocytic development or gametogenesis (Van der Ploeg *et al.* 1985). The striking observation made by PFG analysis of *P. falciparum* chromosomes from different geographical isolates is the extensive chromosomal polymorphism apparent in this organism. Variation in the sizes of chromosomes has been observed to exceed 15% of the total length of an homologous molecule. The origin of such polymorphisms and their significance to the parasite have recently yielded some important insights.

Molecular Basis for Chromosomal Polymorphisms

The determination that homologous chromosomes can vary extensively in size raises the question whether such variations are the result of reciprocal exchanges of DNA between heterologous chromosomes, the result of deletions, or the result of DNA amplification. To address this question, detailed physical maps of polymorphic homologs of chromosomes 1, 2, 4, and 13 have revealed that deletion of sequences does indeed result in the observed differences in the sizes of the polymorphic homologs (Wellems *et al.* 1987; Corcoran *et al.* 1988; Sinnis and Wellems 1988; Patarapotikul and Langsley 1988). It is of particular interest that the deletions are confined to the ends of these chromosomes, making those regions of the chromosome more labile. Attempts to identify the deleted segments elsewhere in the genome have been negative, further supporting the conclusion that interchromosomal exchange is not responsible for the size variations seen. A recent study hypothesized a role for homologous recombination between repetitive sequences (rep 20) in generating the observed chromosomal polymorphisms in chromosomes 1 and 2 (Corcoran *et al.* 1988). As is described below, detailed analysis of chromosome 1, 2, and 8 polymorphisms revealed no basis for homologous recombination in generating these polymorphisms. Rather, chromosome breakage and "healing" by the direct addition of telomere repeats are responsible for these polymorphisms (see below). While not excluded, no evidence has been reported for DNA amplification generating chromosomal polymorphisms.

The deletions that have been characterized can result in the loss of coding as well as in reiterated sequences. In the first such example, a polymorphism of chromosome 2 was indentified as resulting from deletion of the KAHRP gene (Pologe and Ravetch 1986). This results in a parasite which is unable to synthesize the knob structure. The role of the knob is to mediate the interaction of the infected erythrocyte with the endothelial cells lining the postcapillary venules. This interaction is responsible for the sequestration of the infected erythrocyte in the deep vasculature and contributes to the high morbidity and mortality of falciparum malaria. Loss of the knob results in a parasite which is nonviable *in vivo* since it cannot sequester and is cleared by the spleen. Deletion is thus not without its consequences. While the majority of studies have been performed on isolates adapted to *in vitro* culture and maintained in that fashion, chromosomal polymorphisms have been observed in natural infections as well (Corcoran *et al.* 1986). Finally, it appears that stability of a particular karyotype is both strain-dependent and subject to environmental growth conditions. Using clonal isolates, several groups have observed the stability of certain karyotypes upon long-term propagation in culture, with other strains demonstrating instability with the emergence of deletions for chromosomes 2 and 8 (W. Trager, personal communication; Wellems *et al.*, in press). A recent study followed the karyotypes of a clone subjected to mefloquine selection or maintained in the absence of the drug. Both populations developed novel karyotypes,

with the emergence of polymorphisms being more numerous in the parasites exposed to drug selection (Wellems *et al.*, in press). Included among the changes observed was the deletion of the HRP II gene on chromosome 8. Once again, the changes observed appeared to be confined to chromosomal ends.

From the studies cited above it is clear that chromosomal polymorphisms can occur during mitotic growth of the parasite and may reflect the mechanism by which the parasite adapts to changes in environmental states. A prior study (Walliker *et al.* 1987) demonstrated that chromosomal polymorphisms can also arise meiotically In that study, the cross-fertilization of mosquitoes with two distinct strains resulted in the emergence of nonparental karyotypes when analyzed after cloning of the resultant blood stage organisms. Those nonparental karyotypes included the independent assortment of chromosomes in a Mendelian fashion, as well as the generation of novel-sized chromosomes. Analysis of one such novel chromosome demonstrated that crossing-over between homologs was responsible for the novel chromosome (Sinnis and Wellems 1988). Extensive deletions at the ends of these chromosomes did not preclude pairing and exchange. Recombination during the sexual cycle, as occurs during mosquito transmission, is an important component in the emergence of novel karyotypes, but cannot explain the mitotic instability observed, nor the observation that polymorphisms map to the ends of chromosomes.

Chromosome Breakage and Telomere Healing Results in Large Deletions

To begin to address the mechanism of mitotic instability of *P. falciparum* chromosomes and to account for the localization of these deletions to chromosome ends, detailed characterizations of polymorphic homologs of chromosomes 1, 2, and 8 were carried out (Pologe and Ravetch 1988). In all cases, deletion of coding sequences resulted in smaller chromosomes, with loss of RESA expression in the chromosome 1 polymorphism, KAHRP expression in the chromosome 2 polymorphisms, and HRP II expression in the chromosome 8 polymorphism examined. The analysis of these deletions in multiple independent clones revealed that an abrupt transition occurred within coding sequence to simple telomeric repeats. No evidence was found for subtelomeric sequences at these chromosome ends nor was a target for homologous recombination apparent. The structure of these chromosomal polymorphisms is most consistent with a mechanism in which breakage of the chromosome within the coding sequence occurs, followed by the addition of telomere repeats to "heal" the broken ends and stabilize the truncated chromosomes. The orientation of the KAHRP gene on chromosome 2 in K$^-$ isolates, the HRP II gene on chromosome 8 in HRP II$^-$, and the RESA gene on chromosome 1 in RESA$^-$ isolates relative to their respective centromeres further indicates that healing can occur on both broken ends, with the fragment associated with a centromere being mitotically stable and maintained. The sequence analysis of multiple, independent clones revealed that two classes of sites in the parent chromosome are subject to this type of breakage and healing, suggesting that some specificity to this event exists. Further examples are under investigation to determine the extent of this specificity. These studies, then, demonstrate a mechanism whereby large deletions occur and result in polymorphic chromosomes.

Functional Consequences of Chromosomal Polymorphisms

An obvious result of the deletion of sequences is the loss of specific antigens. While there is no evidence to support the notion that such deletions might confer a

selective advantage, directed studies are necessary to investigate this possibility. The suggestion that chromosomal rearrangements increase upon exposure of the organism to environmental pressures raises the possibility that genomic plasticity is among the mechanisms employed by this parasite to adapt to environmental stresses (Wellems *et al.*, in press). However, the loss of specific sequences, like the KAHRP gene, results in parasites unable to sequester. The consequence of this loss, then, is the removal of K^- parasites *in vivo* by the spleen. A mechanism which results in a nonviable phenotype would not be maintained in the absence of other selective pressures. It would appear, then, that loss of specific sequences, like the KAHRP, is a consequence of a mechanism which is maintained for another purpose in the parasite. What selective pressure could be driving the maintenance of such a mechanism of breakage and healing? It is intriguing that the structures of the deletions characterized for chromosomes 1, 2, and 8 are identical to what has been observed in the ciliated protozoans. In that class of organisms, developmentally programmed chromosome breakage and healing occur during differentiation, generating a somatic macronucleus from a germline micronucleus (Blackburn 1986). As a consequence of this fragmentation process, genes become transcriptionally active in the macronucleus. A mutant has been characterized in *P. tetraurelia* in which the loss of a specific surface antigen results from aberrant breakage of the chromosome within a transcriptional unit. This results in deletion of the affected gene so that an abrupt transition is observed between transcribed sequences and telomeric repeats (Forney and Blackburn 1988). The chromosome structure generated is indistinguishable from that described for the chromosome 1, 2, and 8 deletions (Pologe and Ravetch 1988). I would like to suggest that a wild-type counterpart to the mechanism of chromosome breakage and healing may similary exist in *P. falciparum*, analogous to that described in the ciliated protozoans. Repositioning genes in the vicinity of chromosome ends is a general strategy for gene activation (Borst and Greaves 1987) and may be functioning in *P. falciparum* as well. Thus, in the course of the complex life cycle of the parasite the activation of specific genes, or classes of genes, by altering their relationship to a telomere may be involved in their transcriptional regulation. It remains to be seen whether chromosomal polymorphisms can result in the expression of specific sequences, perhaps during switches in life cycle or in response to environmental pressure. In this context, then, the strains which demonstrate genotypic instability of particular chromosomes may provide evidence for the existence of other genetic loci which encode *trans*-acting products required for the breakage and healing events described above. Mutations in these loci could result in the inappropriate breakage or healing of chromosomes to yield some of the karyotypes observed.

The tantalizing glimpses of the mechanisms operating in this parasite to result in its enormous genetic plasticity suggest the directions for future study. Perhaps most important is the frequency at which these novel karyotypes appear in wild populations. The application of the polymerase chain reaction to the analysis of rare events will circumvent the previous difficulties experienced in the analysis of *in vivo* populations. How the parasite avoids accumulating progressively shorter chromosomes as a consequence of the mechanisms described here will need to be addressed. The functional consequences of these polymorphisms are just now being addressed and should yield important insights into the biology of the parasite. Finally, with such information being available, rational approaches to the intervention in the disease caused by this important pathogen may be possible.

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